

**Sperm morphology and function in passerine birds:
insights from intra- and interspecific studies**

by

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We have already started the new sperm project, “Sperm, speciation and promiscuity – an integrated analysis of evolution in passerine birds”, here at NHM, and the first field season of the project is actually finished. I would like to take the opportunity to thank Eduardo, Jostein and Even for a great first field season and also welcome Melissah to our group. I am sure we all have some fruitful scientific years ahead.

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List of publications included in the thesis

The thesis consists of the following five papers, which will be referred to by the Roman numerals given below:

- I. **Laskemoen T**, Fossøy F, Rudolfson G & Lifjeld JT. 2008. Age-related variation in primary sexual characters in a passerine with age-related fertilization success, the bluethroat *Luscinia svecica*. *Journal of Avian Biology* **39**: 322-328
- II. **Laskemoen T**, Kleven O, Fossøy F & Lifjeld JT. 2007. Intraspecific variation in sperm length in two passerine species, the bluethroat *Luscinia svecica* and the willow warbler *Phylloscopus trochilus*. *Ornis Fennica* **84**: 131-139
- III. Kleven O, **Laskemoen T**, Fossøy F, Robertson RJ & Lifjeld JT. 2008. Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution* **62**: 494-499
- IV. Kleven O, Fossøy F, **Laskemoen T**, Robertson RJ, Rudolfson G & Lifjeld JT. 2009. Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution* **63**: 2466-2473
- V. **Laskemoen T**, Kleven O, Fossøy F, Robertson RJ, Rudolfson G & Lifjeld JT. 2010. Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*. *Behavioral Ecology and Sociobiology* **64**: 1473-1483

Abstract

Sperm cells are the most variable animal cells, and a tremendous variation in sperm phenotypes exists among species, from minute amoeboid sperm to giant sperm in some species of fruit flies. Much effort has been devoted to the study of sperm evolution. Sperm competition, when sperm from two or more males compete over fertilization of a set of ova, is thought to be the major force driving the evolution of sperm. There are several ways in which sperm competition can effect the evolution of sperm traits, e.g. through increased sperm production, sperm motility and size.

This thesis concerns variation among and function of sperm in passerine birds. At three levels, within males, between males of the same species and between males of different species, co-workers and I have studied variation in sperm morphology and motility of selected species of passerine birds. Sperm traits, such as sperm numbers, sperm size and sperm motility are potentially important for male fertilization success. Hence, sperm traits are expected to be under strong selection due to evolutionary forces such as e.g. sperm competition. Thus, it is surprising how scarce the available information on sperm traits (size, variation, motility) is in passerine birds.

In an intraspecific study on bluethroats *Luscinia svecica*, we found that primary sexual characters, but not sperm size or motility, were age-dependent. These results suggest that increased sperm production in older males can help explain the age-dependent patterns in fertilization success observed in this species. Further, we documented that between-male variation in sperm size is higher than within-male in bluethroats and willow warblers *Phylloscopus trochilus*, and through resampling procedures we suggested appropriate sample sizes for spermatozoa measured per male and number of males that should be sampled to obtain precise estimates of sperm size and sperm size variation. Moreover, we found that variation in sperm size is closely and negatively related to risk of sperm competition, in a comparative analysis of 22 passerine species. Our interpretation of these results is that increased risk of sperm competition leads to stabilizing selection on sperm size. In another comparative study on 42 passerine species, we documented that sperm swimming speed and sperm length is positively related to risk of sperm competition. However, we found no relationship between sperm swimming speed and sperm size. Thus,

it seems that sperm competition leads to both increased sperm swimming speed and increased sperm length, independent of each other. In addition, sperm swimming speed was negatively related to clutch size, a proxy for female sperm storage. Finally, we found in an intraspecific study on tree swallows *Tachycineta bicolor*, that both sperm quantity and quality were significantly related to fertilization success. This result has great implications as the first study linking sperm traits and fertilization success in a free living passerine.

General introduction

The primary function of sperm cells is to fertilize eggs, but despite this universal purpose, there is a large diversity across the animal kingdom in sperm phenotypes. Much effort has been devoted to the study of sperm evolution (Birkhead *et al.* 2009), in particular the effect of sperm competition on sperm quantity and quality traits (Parker 1970; Simmons 2001; Snook 2005; Briskie & Montgomerie 2007). Sperm competition occurs when sperm from two or more males attempt to fertilize the same set of ova (Parker 1970) and is widespread throughout the animal kingdom (e.g. Birkhead & Møller 1998). There are several ways in which sperm competition can affect the evolution of sperm traits, e.g. through the selective advantages of increased sperm production, sperm motility and sperm size (Parker 1998; Snook 2005). Higher sperm production may allow males to copulate more often and/or release more sperm per ejaculate, and increased sperm size and motility might enhance individual male's competitive ability after copulation (e.g. Parker 1993; Parker 1998). Postcopulatory sexual selection includes both male sperm competition and cryptic female choice, i.e. females biasing the fertilization success of the males that inseminate them (Eberhard 1996; Birkhead & Pizzari 2002). Cryptic female choice may in effect counteract the influence of male sperm traits on fertilization success and open for sexual conflict over fertilizations (Stockley 1997).

Several comparative studies have been conducted on the relationship between sperm competition and sperm traits. In mammals, Gomendio & Roldan (1991) found a positive relationship between sperm competition and sperm size, and a positive relationship between sperm size and sperm motility. Similar relationships have been shown in moths (Gage 1994), fish (Balshine *et al.* 2001), birds (Briskie & Montgomerie 1992; Briskie *et al.* 1997; Johnson & Briskie 1999), and mammals again (Gomendio & Roldan 2008). However, some studies have yielded mixed (Immler & Birkhead 2007), no (e.g. Hosken 1997; Gage & Freckleton 2003; Immler *et al.* 2007), or negative (Stockley *et al.* 1997) relationships between sperm competition and sperm size. In an intraspecific study, males of the cricket *Gryllus bimaculatus* producing relatively small sperm and relatively numerous sperm won in the competition for fertilization (Gage & Morrow 2003).

Sperm cells are the most variable of all animal cells with respect to size (Cohen 1977). The variability in size and morphology spans from the minute amoeboid sperm of nematodes and mite (Radwan 1996; LaMunyon & Ward 1998) to the almost 60 mm spermatozoon of the fruit fly *Drosophila bifurca* (Diptera: Drosophilidae) (Pitnick *et al.* 1995). In birds, the variation is lower, but still ranges almost seven-fold, from 43 μm in the red-backed shrike *Lanius collurio* (Briskie *et al.* 1997) to 291 μm in the reed bunting *Emberiza schoeniclus* (Dixon & Birkhead 1997). Figure 1. illustrates some of the large sperm size variation in a selection of passerine birds.

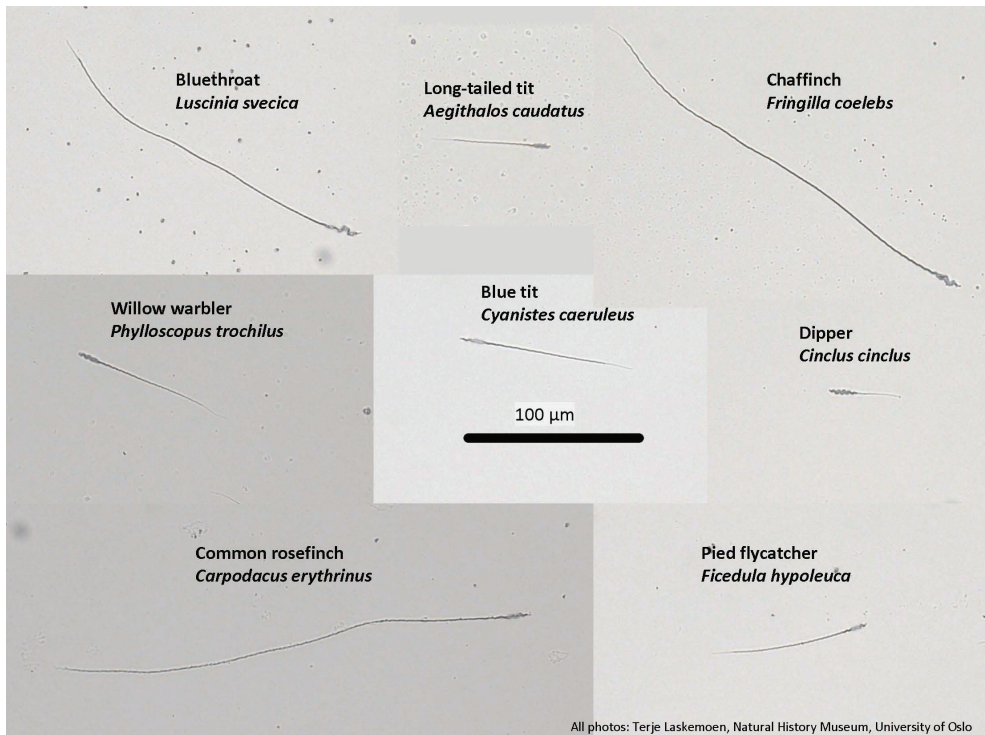


Fig. 1. Digital photos of spermatozoa from a selection of passerine species, illustrating the large variation in sperm size and morphometry across species. All photos are scaled to the 100 μm scale bar in the centre. All photos taken with a Leica DFC420 camera mounted onto a Leica DM6000 B digital microscope.

The large interspecific variation in sperm size has been well described in many taxa, e.g. insects (Gage 1994), fish (Balshine *et al.* 2001; Fitzpatrick *et al.* 2009), mammals (Gomendio & Roldan 1991; Gage 1998; Gage & Freckleton 2003), and birds (Koehler

1995; Johnson & Briskie 1999; Briskie & Montgomerie 2007). However, Ward (1998) pointed out that far less is known about intraspecific variation in sperm length and other sperm traits. This is unfortunate since the intraspecific variation in an important statistical parameter when examining differences in sperm length between species, for example in comparative analyses, and the degree of intraspecific variation may be an important feature itself, shaped by selection or other evolutionary forces (Birkhead *et al.* 2005).

Passerine sperm are distinct in morphology as compared to sperm of other taxa. The passerine spermatozoa are characterized by having a helical shaped head, containing the acrosome and nucleus, a helical shaped mitochondrion wrapped around the flagellum, and a straight tail distally on the flagellum (Koehler 1995) (Fig. 2).

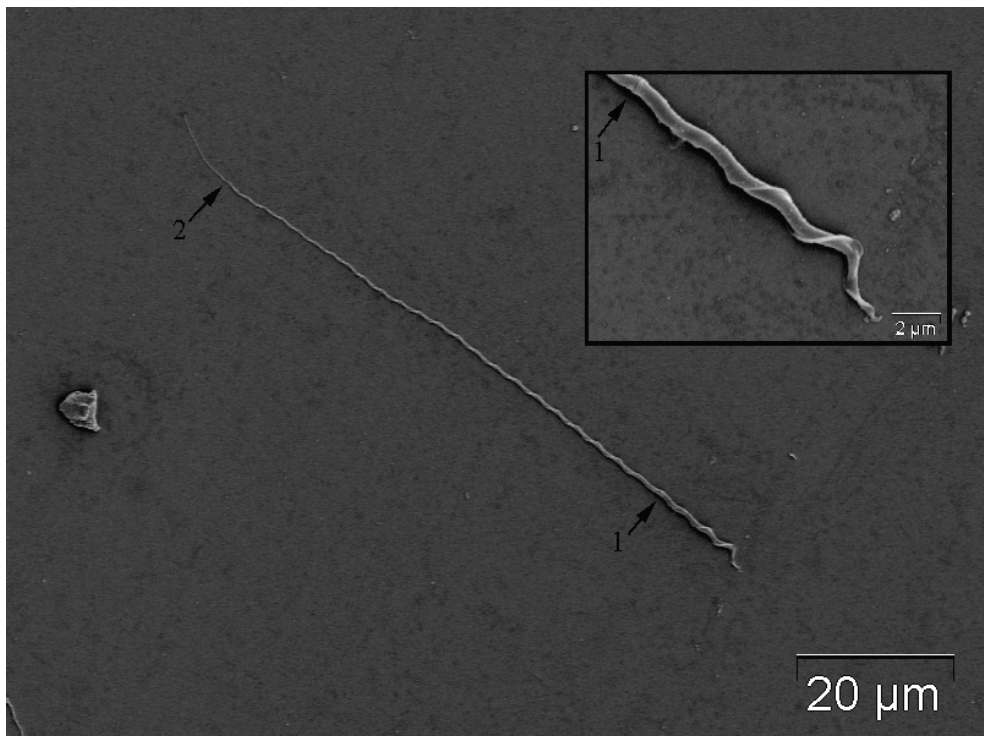


Fig. 2. Scanning electron micrograph of a willow warbler *Phylloscopus trochilus* spermatozoon, illustrating the characteristic helical shaped head and midpiece, and the straight tail. The arrows indicate (1) the limit between head and midpiece, and (2) the limit between midpiece and tail.

Also, passerine sperm are quite different in swimming behavior as compared to other flagellate sperm, both close and distant taxa (Vernon & Woolley 1999). The sperm swim

by spinning around their own axis (Vernon & Woolley 1999), and not by solely beating their flagellum, as for example mammalian sperm.

The aims of this thesis were to investigate sperm morphology and function in passerine birds at three levels, within males, between males of the same species, and comparatively between males of different species. More specifically, we wanted to test whether sperm competition, as a selective force, influences sperm size, variation in sperm size and sperm motility in passerine birds. The predictions here are that increased risk of sperm competition will lead to (1) increased sperm size, (2) decreased variation in sperm size, both within and between males and (3) increased sperm swimming speed. Further, we aimed to investigate the relative roles of sperm traits, i.e. sperm numbers, sperm morphometry and motility, for fertilization success in a free living passerine. In this context we predict that sperm production (sperm numbers), sperm length and length of the sperm midpiece, as well as sperm swimming speed are positively related to individual males' fertilization success.

In paper I, we investigated variation in primary sexual characters (size of the reproductive organs), sperm size, and sperm motility, with age in the bluethroat *Luscinia svecica*. In paper II, we investigated the intra- and intermale variation in sperm size in bluethroats and willow warblers *Phylloscopus trochilus*. Further, we conducted resampling procedures to assess the number of sperm per male and males per species that is needed to obtain precise estimates of mean sperm size and sperm size variation, within and between males. In paper III, we tested the hypothesis that increased risk of sperm competition leads to reduced variation in sperm size among males, using a comparative approach with 22 species of passerine birds. In paper IV, we tested the hypotheses that sperm swim faster with (1) increased risk of sperm competition, (2) increased sperm length, and (3) shorter duration of female sperm storage, using a comparative approach with 42 free-living passerine species. In paper V, we investigated the associations between sperm traits, quantity and quality, and fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*.

General methods

Field work

Field work was conducted in three main areas, in the vicinity of Queens University Biological Station (44°34' N, 79°19' W), Ontario, Canada, at the Natural History Museums field station in Øvre Heimdalen (61°25' N, 8°52' E), Oppland, Norway, and in southern Norway, mainly nearby Oslo (59°55'N, 10°44' E), Oslo, Norway, during the field seasons (start of April until end of June) of 2002, 2004, 2006 and 2007. Free-living males were captured using mist-nets and playback of conspecific song, or captured in box-traps. To avoid pseudo-replication (i.e. that an individual was sampled twice), the males were banded with a uniquely numbered aluminium band provided by the Canadian Wildlife Service (for birds banded in Canada) and the Norwegian Bird Ringing Centre at Stavanger Museum (for birds banded in Norway).

Sperm was sampled either from dissecting the seminal glomera of collected birds (willow warblers in 2002 and bluethroats in 2004), or by gently massaging the cloacal protuberance to obtain an ejaculate. In passerines, the cloacal protuberance is swollen, as a result of the growth of the seminal glomera (where mature sperm are stored before ejaculation), in males in breeding condition (Wolfson 1952). The ejaculate was picked up using a small (10 µl) micropipette, and fixed in a ~5% formalin solution. Notably, sperm samples tend to clump in pure distilled water/formalin solutions, and we therefore homogenized the sperm sample in a small volume of PBS before fixing it in the formalin solution. None of the birds showed any sign of suffering during sperm sampling, which usually lasted just a few seconds. Except for the birds collected under license from the Norwegian Directorate for Nature Management (DN) in 2002 and 2004, all males were released immediately after banding and sampling.

Sperm motility recordings

After obtaining the ejaculate, it was immediately diluted in 20-100 µl (depending on the size of the ejaculate) preheated (40°C) Dulbecco's Modified Eagle Medium (Advanced D-MEM, Invitrogen, Carlsbad, CA). Within 30 seconds after ejaculation, about 3-5 µl of the diluted sperm was pipetted onto a preheated standard microscope count slide (20 µm depth

two-chamber, Leja, Nieuw-Vennep, The Netherlands) mounted on a MiniTherm slide warmer (Hamilton Thorne Inc, Beverly, MA) kept at a constant temperature of 40°C. Sperm motility was then recorded for up to 90 seconds using a digital video camera (HDR-HC1E, PAL, Sony, Tokyo, Japan) mounted on an upright microscope (CX41, Olympus, Japan) equipped with a 4× objective. Multiple independent frames were recorded for each slide to increase the number of sperm measured for each male.

Sperm motility analyses

Computer assisted sperm analysis (HTM-CEROS sperm tracker, CEROS version 12, Hamilton Thorne Biosciences, U.S.A.) was used to analyze the sperm motility recordings. The sperm analyzer was set at a frame rate of 50 Hz and 25 frames (i.e. sperm cells were tracked for 0.5 s). Each analysis was visually examined and cell detection parameters were adjusted using the two interactive quality control plots as well as directly from visual examination of each recording. Cell detection parameters thus varied slightly; minimum contrast 78.3 ± 10.2 SD and minimum cell detection size 12.2 ± 0.63 SD pixels. We recorded the average percentage motile sperm, average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL), weighed by number of motile spermatozoa in the different frames, for each male. In order to remove a potential effect of drift in the chamber, spermatozoa with VAP less than 10 $\mu\text{m/s}$ and VSL less than 5 $\mu\text{m/s}$ were counted as static and excluded from the swimming speed analysis. Sperm cell trajectories were not expected to be linear, since there was no egg or other components in the D-MEM to attract or guide the spermatozoa (cf. Eisenbach & Giojalas 2006). We therefore used the actual point-to-point track (VCL) as our measurement of sperm swimming speed. To avoid inter-observer effects, all sperm motility analyses were conducted by one person (G. Rudolfsen), and blindly with respect to male identity.

Sperm morphometry measurements

All sperm samples were fixed in tubes containing a ~5% formalin solution, except for the sperm samples of willow warblers and bluethroats from 2002 and 2004, which were fixed in tubes containing a ~2% glutaraldehyde solution. Sperm slides were prepared by placing

a droplet of the fixed sperm on a microscope slide, which was allowed to air dry. Sperm were examined using a digital light microscope (Leica DM6000 B) at magnifications of 160 \times , 200 \times and 320 \times , depending on the sperm size, and pictures were taken using a digital camera mounted on the microscope. We used either the line-chain tool in the Leica IM100 software to measure the total sperm length ($\pm 0.1 \mu\text{m}$), from the anterior tip of the acrosome to the posterior end of the flagellum, on the digital images (paper I, II and III), or the line-chain tool in the Leica Application Suite (Version 2.6.0 R1) to measure the length ($\pm 0.1 \mu\text{m}$) of the head, midpiece and tail (paper IV and V). Total sperm length was calculated as the sum of head, midpiece and tail, and flagellum length was calculated as the sum of midpiece and tail. The initial camera (Leica DC500) and software (Leica IM1000) were replaced by a new camera (Leica DCF420) and new software (Leica Application Suite ver. 2.6.0 R1) in 2008, and hence, this part of the methods section in the papers vary accordingly. All measurements of sperm size were performed by one person (T. Laskemoen), to control for potential observer effects.

Statistical analyses

Statistical analyses were carried out using several different statistical programs, Statistica versions 6.1 and 7.1 (StatSoft, Inc), Resampling Stats for Excel (Resampling Stats, Inc), SPSS version 14.0 (SPSS, Inc), and R versions 2.5.1 and 2.6.0 (R Development Core Team 2007). For the comparative papers (III and IV), we used a general least-squares (GLS) approach to control for potential non-independence in the data as a result of shared ancestry (Pagel 1999; Freckleton *et al.* 2002). Briefly, the GLS method estimates the degree of phylogenetic correction required for comparative data. Through a maximum-likelihood framework, an index of phylogenetic association, λ , is estimated, with values ranging from 0 (indicating phylogenetic independence) to 1 (indicating complete phylogenetic dependence). Likelihood-ratio tests are used to compare whether the estimated model λ differs from models with λ values of 0 (phylogenetic independence) or 1 (phylogenetic dependence). Phylogenies of the species included in the analyses were based on recent molecular phylogenies (see supplementary information paper III and IV). The analyses were performed in R using the package APE and a script kindly provided by R. P. Freckleton

Summary of publications

Paper I

Age-related variation in primary sexual characters in a passerine with male age-related fertilization success, the bluethroat *Luscinia svecica*

Several studies have showed that older males are more successful in achieving extrapair fertilizations as compared to young males. However, the explanation behind this pattern is not clear. In this study, we tested the hypothesis that older males (after second year) had larger primary sexual characters (testes, seminal glomera and cloacal protuberance) than young (second year males). In addition, we tested whether sperm size and velocity differed between the two age groups. Other studies have found age-related variation in testis size, which we also showed in bluethroats. In addition, we found that older males also had significantly larger seminal glomera and cloacal protuberance than young males, indicating higher rate of sperm production in older males. Sperm size and sperm velocity did not differ between the two age groups. Unequal sperm production capacities may explain age-related fertilization success in bluethroats, and also have important implications for the interpretation of paternity patterns in extrapair mating systems.

Paper II

Intraspecific variation in sperm length in two passerine species, the bluethroat *Luscinia svecica* and the willow warbler *Phylloscopus trochilus*

It is well known that sperm size and morphology vary tremendously between species and taxa. Far less is known about variation in sperm size within species. In this study, we analyzed within- and between-male variation in total sperm length in two passerine species, the bluethroat and the willow warbler. Both species are characterized by showing relatively high levels of sperm competition, as measured by levels of extrapair paternity. In both species, the variance in total sperm length was approximately twice as high between

as within males, resulting in high repeatability of total sperm length for individual males. We conducted two resampling approaches, first to illustrate how the spread in estimated mean sperm length and coefficient of variation (CV) is affected by increasing the number of males measured, and second, to illustrate how the CV of sperm length for individual males is affected by increasing the number of spermatozoa measured. For the two species in our study, measuring 10 males and at least 10 spermatozoa per male seems to give adequate estimates of both within- and between-male sperm length and CV.

Paper III

Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds

Sperm size and morphology are likely to be under selection due to sperm competition. Another sperm trait that could be under selection due to sperm competition is sperm size variation. In this context, increased sperm competition is thought to lead to stabilizing selection for lower variation in sperm size. Hence, in this study, we tested the hypothesis that increased risk of sperm competition leads to lower intraspecific variation in sperm size. We examined the relationship between intraspecific variation in sperm length of 22 free-living passerine species (representing 21 genera and 11 families) and the risk of sperm competition, as expressed by the frequency of extrapair paternity and relative testis size. Using comparative methods controlling for phylogeny, we demonstrate that between-male variation in sperm length within species is closely and negatively related to the risk of sperm competition. The relationship was even stronger when only considering species in which data on sperm length and extrapair paternity originated from the same population. Notably, the intraspecific variation in sperm length was more closely related to the frequency of extrapair paternity than to relative testis size. Our results suggest that postcopulatory sexual selection is a powerful evolutionary force reducing the intraspecific phenotypic variation in sperm-size traits.

Paper IV

Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage in passerine birds

Sperm swimming speed is thought to be an important sperm trait linked to fertilization success. However, not much empirical data is available on sperm swimming speed in passerine birds. In addition, little is known about sperm swimming speed variation among bird species. In this study, we tested the hypotheses that spermatozoa swim faster with (1) increased risk of sperm competition, (2) shorter duration of female sperm storage, and (3) increased sperm length. We recorded sperm swimming speed *in vitro* in 42 North American and European free-living passerine species, representing 35 genera and 16 families. Using phylogenetic comparative methods, we found that sperm swimming speed was positively related to the frequency of extrapair paternity (a proxy for the risk of sperm competition) and negatively related to clutch size (a proxy for female sperm storage). We found no evidence for increased sperm swimming speed with increased sperm length, although sperm length was positively related to the frequency of extrapair paternity. These results suggest that sperm swimming speed and sperm length are not closely related traits, and evolve independently in response to sperm competition in passerine birds. Also, our findings emphasize the significance of both sperm competition and female sperm storage duration as evolutionary forces on sperm swimming speed.

Paper V

Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow

Sperm traits, such as motility and numbers, have been shown to be positively associated with fertilization success in a few species. However, in free living passerines, the associations between both sperm quantity and quality and fertilization success have never been tested. Here, we examined the influence of sperm quantity and quality on fertilization success in the socially monogamous, but highly promiscuous tree swallow *Tachycineta*

bicolor. Female tree swallows actively accept or reject male copulation attempts, and have been shown to increase offspring heterozygosity through extrapair fertilizations. Thus it is not intuitively clear that male intrinsic sperm traits should affect fertilization success. We found that male fertilization success (the sum of withinpair and extrapair young) was significantly related to the size of the cloacal protuberance (a proxy for sperm production), but not to *in vitro* sperm swimming speed. However, males with large relative sperm midpiece size (i.e. high mitochondrial loading) had both higher fertilization success and faster swimming sperm, suggesting an indirect link between sperm morphology and male fertility mediated through sperm velocity. In a multivariate analysis, both cloacal protuberance volume and relative sperm midpiece size had significant effects on fertilization success. These results suggest that in tree swallows, both quantitative and qualitative sperm traits are important determinants of fertilization success.

General discussion

This thesis has dealt with sperm morphology and function in passerine birds at three different levels, within males of the same species, between males of the same species and comparatively between species. In an intraspecific study of bluethroats *Luscinia svecica*, we showed that primary sexual characters (size of testes, seminal glomera and cloacal protuberance) were age-dependent, whereas sperm length and sperm motility showed no age-related patterns. In a study of two passerine species, bluethroats and willow warblers *Phylloscopus trochilus*, we found low levels of both within-male variation and between-male variation in sperm size, with the within-male variation being considerably lower than the between-male variation. Using a comparative approach including 21 species of passerine birds, we found a strong negative relationship between risk of sperm competition and intra-specific sperm length variation. We interpret this result as evidence of stabilizing selection on sperm size when the risk of sperm competition increases. In another comparative study of 42 passerine species, we found/showed that sperm swimming speed was positively related to extrapair paternity (a proxy for risk of sperm competition) and negatively related to clutch size (a proxy for female sperm storage). Sperm swimming speed was not related to sperm length, although sperm length also was related to extrapair paternity. This suggests that sperm swimming speed and sperm length evolve independently in response to sperm competition. Finally, we found, in an intraspecific study of tree swallows *Tachycineta bicolor*, that both sperm quantity (size of the cloacal protuberance) and quality (relative midpiece size) was positively related to fertilization success.

Promiscuity is widespread in socially monogamous species, and thus extrapair paternity is common in many species (Griffith *et al.* 2002; Westneat & Stewart 2003). Bluethroats show high levels of extrapair paternity, encompassing about one third of all offspring and about half of all broods (Krokene *et al.* 1996; Johnsen & Lifjeld 2003). Male age has been documented as the strongest predictor of extrapair fertilization success in this species, whereas there is no such age effect on withinpair fertilization (Johnsen *et al.* 2001). Similar patterns, with older males achieving more extrapair fertilizations than young males, have been documented in several other species (Weatherhead & Boag 1995;

Wetton *et al.* 1995; Dunn & Cockburn 1999; Richardson & Burke 1999; Dickinson 2001). We investigated variation in primary sexual characters, sperm size and sperm motility with age in bluethroats (paper I). Older males, after second year, had significantly larger testes, seminal glomera and cloacal protuberances compared to young males, second year, suggesting higher sperm production in older males. Sperm length and motility however, did not differ between the two age classes. As sperm traits have been shown to be heritable in other species (Ward 2000; Birkhead *et al.* 2005; Baer *et al.* 2006), lack of age differences in sperm traits might not be very surprising. We suggest that sperm quantity, and not sperm quality, might explain the pattern of age dependent fertilization success observed in bluethroats (paper I), and other species.

The high variation in sperm size between species has been well studied and documented in several studies (e.g. Gomendio & Roldan 1991; Briskie & Montgomerie 1992; Gage 1994; Briskie *et al.* 1997; Balshine *et al.* 2001). However, as pointed out by Ward (1998), with a few exceptions (e.g. Morrow & Gage 2001), much less is known about the level of intraspecific variation in sperm size. We investigated the levels of intraspecific variation in sperm length in two passerine species with relatively high levels of sperm competition, the bluethroat and the willow warbler, and in general, sperm length was more variable between males compared to within males (paper II). We obtained estimates of intraspecific variation in sperm length from more species and observed a strong pattern, the species coefficient of variation (CV) in total sperm length was closely and negatively related to risk of sperm competition, as measured by levels of extrapair paternity. This lead us to test the hypothesis proposed by Birkhead *et al.* (2005), that increased risk of sperm competition leads to reduced variation in sperm size. In a comparative study of 22 passerine species, controlling for phylogeny, we found that intraspecific variation in sperm size was negatively related to risk of sperm competition (paper III). While focusing on levels of extrapair paternity as a proxy for the risk of sperm competition, we also conducted the analyses using relative testis mass as a proxy for the risk of sperm competition. Although we found a negative relationship between intraspecific variation in sperm size and relative testis mass, the relationship was weaker than the latter relationship between sperm size variation and extrapair paternity. Information on levels of extrapair paternity are available for a wide range of bird species (Griffith *et al.* 2002), and are likely to represent appropriate estimates of the true level of

sperm competition risk (cf. Briskie *et al.* 1997). Relative testis has been more commonly used as a proxy for the risk of sperm competition (e.g. Hosken 1997; Dunn *et al.* 2001; Gage & Freckleton 2003), but should be used with care, as many available estimates are of uncertain quality (Calhim & Birkhead 2007). In another study on passerine birds, Calhim *et al.* (2007) documented a similar negative relationship between sperm size variation and risk of sperm competition, suggesting that this is a general pattern in passerine birds. We also tested the relationship between intramale variation in sperm size and risk of sperm competition, and found a relationship in the predicted direction, although not statistically significant. Recently, such a negative relationship between intramale variation in sperm size and risk of sperm competition was also demonstrated in another study of passerine birds (Immler *et al.* 2008).

Sperm motility seems to be a particularly important determinant of male fertility and postcopulatory competitive ability (reviewed in Snook 2005; Gomendio *et al.* 2007). In fact, sperm motility and/or mobility has been found as significant predictors of fertilization success in some species (e.g. Birkhead *et al.* 1999; Donoghue *et al.* 1999; Froman *et al.* 1999; Gage *et al.* 2004). Other studies have demonstrated that males in inferior roles, such as sneakers or subordinates, produce sperm that swim faster than males in superior roles, such as territory holders or dominants (Burness *et al.* 2004; Rudolfson *et al.* 2006; Fitzpatrick *et al.* 2007). Also, recent comparative studies have revealed that sperm swimming speed increases with the risk of sperm competition across four species of primates (Nascimento *et al.* 2008; not controlling for phylogenetic relatedness) and within a single family (Cichlidae) of externally fertilizing fish (Fitzpatrick *et al.* 2009). We tested the hypothesis that increased risk of sperm competition leads to increased sperm swimming speed, and found support for this in a comparative study of 42 passerine species (paper IV). In addition, sperm swimming speed was negatively related to clutch size, a proxy for duration of female sperm storage (Briskie *et al.* 1997). Although sperm length was positively related to risk of sperm competition, we found no association between sperm size and sperm swimming speed, suggesting that sperm swimming speed and sperm size evolve independently in relation to risk of sperm competition (paper IV). However, Lüpold *et al.* (2009a) found support for a positive relationship between sperm size and sperm velocity. Notably, few species overlapped, and this could help explain the discrepancy between the two studies (Lüpold *et al.* 2009a; paper IV). A comparative study of one

family (Icteridae) of passerine birds revealed support for increase in sperm size with increased postcopulatory sexual selection, but found no relationship between sperm size and motility (Lüpold *et al.* 2009b). Taken together, these results demonstrate that no general pattern between the risk of sperm competition and sperm traits, and among different sperm traits (size and motility) are found in birds (Lüpold *et al.* 2009a; Lüpold *et al.* 2009b; paper IV), which may partly be explained by phylogenetic constraints (Immler & Birkhead 2007).

Intraspecific studies have found significant effects of sperm traits on fertilization success (e.g. Donoghue *et al.* 1999; Froman *et al.* 1999; Denk *et al.* 2005), and males of domestic fowl *Gallus gallus* have been found to be able to strategically allocate ejaculates (Pizzari *et al.* 2003). In sand martins *Riparia riparia*, sperm length was negatively related to initial sperm velocity, but positively related to sperm longevity (Helfenstein *et al.* 2008). However, the relationships revealed in Helfenstein *et al.* (2008), is likely to reflect sperm midpiece size rather than sperm total length (Kleven *et al.* 2009). Still, this relationship is interesting and in support of the hypothesis that increased midpiece size may lead to higher energy loading, and thus increased longevity (Cardullo & Baltz 1991). In a population of free-living tree swallows, both cloacal protuberance volume (a proxy for sperm production) and relative sperm midpiece size predicted total fertilization success, and both these predictors were positively related to sperm velocity (paper V). Cloacal protuberance volume also predicted number of extrapair young sired, but we found no evidence of any sperm traits predicting withinpair paternity. In another study on copulation behavior and paternity, it was demonstrated that social tree swallow males can secure withinpair paternity by copulating more often with their social female (Crowe *et al.* 2009). The results in paper (V) demonstrates that, in a passerine species where females are in, at least some, control of copulations (Lifjeld & Robertson 1992), and females have been found to increase offspring heterozygosity through extrapair mating (Stapleton *et al.* 2007), male sperm traits play an important role in fertilization success.

In summary, the papers included in this thesis have contributed to our knowledge about sperm evolution in passerine birds. We have shown that sperm size varies consistently both within individuals, between males of the same species and between species. Both the between-male variation and the species specific within-male variation in sperm size are negatively related to risk of sperm competition. In fact, we have now

increased the data set of species with available information on extrapair paternity and estimations on sperm size variation to 55 species, and the relationship between extrapair paternity and sperm size variation is still negative and highly significant (Lifjeld JT, Laskemoen T, Kleven O, Albrecht T & Robertson RJ unpublished data). As the levels of extrapair paternity rates may vary considerably between populations (e.g. Griffith *et al.* 2002), we also restricted the data set to species where data on extrapair paternity and sperm size variation originated from the same population, and found an even stronger relationship ($n=24$ species, $r^2=0.66$, $p<0.001$). The implications of this result is that sampling a few males, ideally 10 or more, of a given species, and estimating the between-male variation in sperm size, enables one to give a good estimation of the species' level of sperm competition. This relationship remains to be examined in other groups of birds and other animal taxa. Further, we have shown that the risk of sperm competition is positively related to sperm swimming speed comparatively across passerines, suggesting that sperm competition is an important selective force, driving the evolution of sperm swimming speed. Finally, we demonstrated, in an intraspecific study of tree swallows, that both sperm quantity and quality traits were associated with male fertilization success. These associations remains to be tested in a larger number of species before one can claim any general consistence between sperm traits and fertilization success.

Future prospects

Another possibly important sperm characteristic is sperm longevity. So far, there is a lack of studies examining the role of sperm longevity for fertilization success in birds. One exception is a study on mallards *Anas platyrhynchos*, which indicated that sperm motility 8 minutes post ejaculation was one of the best predictors of fertilization success (Denk *et al.* 2005). The general lack of studies on sperm longevity is unfortunate since birds store sperm over relatively long periods, and sperm longevity may actually be a key determinant of, for example, fertilization success. Hence, there is a need for studies investigating sperm longevity in relation to risk of sperm competition and fertilization success both in birds and other taxa.

While emphasizing that sperm traits (both quantity and quality) are important determinants of fertilization success, one must not neglect the female factors due to postcopulatory sexual selection (e.g. Eberhard 1996). There is an increasing body of literature indicating that genetic relatedness or genetic similarity between mating partners influences competitive fertilization success (e.g. Stockley 1999; Mack *et al.* 2002; Bretman *et al.* 2004; Thuman & Griffith 2005; Jehle *et al.* 2007). This calls for intraspecific studies of sperm traits and fertilization success controlling for genetic relatedness or genetic similarity of the individuals. We have some data indicating that sperm swimming speed is lower in fluid from more genetically similar females as compared to less genetically similar females in the willow warblers (Kleven O, Johnsen A, Rudolfson G, Laskemoen T, Øigarden T & Lifjeld JT unpublished data). The mechanism behind this possible cryptic female choice could be interactions between proteins from the male ejaculate and the female tract, but this needs further investigation. In summary, sperm evolution is still poorly understood in many contexts, and more questions will be answered as more sophisticated techniques are developed.

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Intraspecific variation in sperm length in two passerine species, the Bluethroat *Luscinia svecica* and the Willow Warbler *Phylloscopus trochilus*

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Sperm cells are highly diversified in birds and considerable research effort has focused on variation in sperm morphology between species. However, surprisingly little is known about intraspecific variation in sperm morphology in birds. We analyzed between- and within-male variation in total sperm length in two passerine species, the Bluethroat (*Luscinia svecica*) and the Willow Warbler (*Phylloscopus trochilus*). In both species, the variance in sperm length was nearly twice as high between as within males, resulting in high repeatability of sperm length for individual males (Bluethroat: $r = 0.73$ and Willow Warbler: $r = 0.79$). These results suggest that sperm traits are more variable among than within males. With a resampling approach, we illustrate how the spread in estimated mean sperm length and coefficient of variation (CV) is affected by increasing the number of males measured. Further, we illustrate how the CV of sperm length for individual males change with the number of spermatozoa measured. For the two species in our analyses, it seems that measuring 10 males and 10 spermatozoa per male gives adequate estimates of both between- and within-male sperm length and CV.



1. Introduction

Spermatozoa are by far the most diverse cells in the animal kingdom (Cohen 1977). Although spermatozoa generally are numerous and tiny, there is enormous variation in sperm length across animal taxa, ranging from 15.5 μm in the cichlid *Asprotilapia leptura* (Perciformes: Cichlidae) (Balshine *et al.* 2001) to 58,290 μm in the fruit fly

Drosophila bifurca (Diptera: Drosophilidae) (Pitnick *et al.* 1995). In birds, the variation is lower, but still ranges almost seven-fold, from 42.7 μm in the Red-Backed Shrike (*Lanius collurio*) (Briskie *et al.* 1997) to 291 μm in the Reed Bunting (*Emberiza schoeniclus*) (Dixon & Birkhead 1997). This huge interspecific variation in sperm length is well described in many taxa, including birds (e.g. Koehler 1995, Briskie & Montgomerie 2007).

Table 1. Recent publications (1992–present) addressing sperm size in birds. Indicating number of species, number of males per species and how many spermatozoa measured per male.

| Species | Number of species | Males per species | Spermatozoa measured | Reference |
|-------------------------|-------------------|-------------------|----------------------|-------------------------------|
| Passerines | 20 | 1–5 | 10 | (Briskie & Montgomerie 1992) |
| Zebra Finch | 1 | 10 | 50 | (Birkhead & Fletcher 1995) |
| Passerines | 13 | NA ¹ | NA | (Koehler 1995) |
| Fairy Wrens | 3 | 17–52 | NA | (Tuttle <i>et al.</i> 1996) |
| Sedge Warbler | 1 | 14 | 30 | (Birkhead <i>et al.</i> 1997) |
| Passerines | 21 | 1–5 | 10 | (Briskie <i>et al.</i> 1997) |
| Reed Bunting | 1 | 4 | 30 | (Dixon & Birkhead 1997) |
| Shorebirds | 16 | 1–3 | 5–10 | (Johnson & Briskie 1999) |
| Zebra Finch | 1 | 913 | 5 | (Birkhead <i>et al.</i> 2005) |
| Passerines ² | 37 | 1–33 | 10 | (Immler & Birkhead 2005) |
| Passerines | 21 | 1–11 | 5, 15 and 30 | (Birkhead <i>et al.</i> 2006) |
| Passerines | 18 | 10–236 | 5 | (Calhim <i>et al.</i> 2007) |
| Passerines | 73 | 1–10 | 5 | (Immler & Birkhead 2007) |
| Pheasants | 24 | 1 | 15 | (Immler <i>et al.</i> 2007) |

1. NA = data not available.

2. One non-passerine species.

However, Ward (1998) pointed out that far less is known about the level of intraspecific variation in various sperm traits. This is unfortunate for two main reasons. First, intraspecific variation is an important statistical parameter when examining differences in sperm length between species, as for example, in comparative analyses. Second, the degree of intraspecific variation may be an important feature itself which may be shaped by selection or other evolutionary forces (Birkhead *et al.* 2005).

Much of the variation in sperm length can be attributed to phylogenetic relatedness, but some comparative studies, controlling for phylogeny, have indicated that there is a positive association between the risk of sperm competition and sperm length in insects (Gage 1994), fish (Balshine *et al.* 2001), mammals (Gomendio & Roldan 1991) and birds (Briskie & Montgomerie 1992, Briskie *et al.* 1997, Johnson & Briskie 1999). However, more recent studies of mammals (Gage & Freckleton 2003) and birds (Immler & Birkhead 2007) found no support for the positive relationship between risk of sperm competition and sperm length. In this context, especially when comparing closely related taxa, it is important to have adequate estimates of means and their variances.

In taxa other than birds, quite a few studies have followed Ward's (1998) recommendations and addressed intraspecific variation in sperm

length (e.g. Morrow & Gage 2001, Joly *et al.* 2004, Schulte-Hostedde & Millar 2004, Bernasconi & Hellriegel 2005, Hettyey & Roberts 2006, Malo *et al.* 2006, Minoretti & Baur 2006, Schulte-Hostedde & Montgomerie 2006, Harris *et al.* 2007, Locatello *et al.* 2007), but such studies are still missing in birds. With a few notable exceptions (Tuttle *et al.* 1996, Dixon & Birkhead 1997, Birkhead *et al.* 2005, Birkhead *et al.* 2006), the variance or standard error of the means are usually not reported. These shortcomings call for an evaluation of intraspecific variation in sperm length in birds, especially since birds to a great extent are subject to evolutionary and ecological studies of mating systems, in which various aspects of sperm biology might play an important role.

As part of ongoing studies of infertility and testis size variation in the Bluethroat (*Luscinia svecica*) and the Willow Warbler (*Phylloscopus trochilus*) in Southern Norway (Lifjeld *et al.* 2007, Laskemoen *et al.* in press), we also collected sperm samples from breeding males of both species. In the present study, we quantify the between- and within-male variation in sperm length in these species. As sample sizes (number of males sampled) vary considerably between studies (see Table 1), we wanted to examine how the spread in estimated mean sperm length and coefficient of variation (CV) is influenced by increasing the number

of males measured. In order to do so we apply resampling procedures on mean sperm length (1–30 males) and CV of sperm length (2–30 males). Further, the numbers of spermatozoa measured per male vary to a great extent in recently published studies (Table 1). Therefore, we present resampling estimates on within-male CV of sperm length (2–30 spermatozoa) as a function of the number of spermatozoa measured.

2. Material and methods

2.1. Study area and species

Field work was carried out in the valley Øvre Heimdalen (61°25'N, 8°52'E), Øystre Slidre municipality, Oppland county, Norway, during two field seasons (2002 and 2004). The study area is located at an altitude of about 1,100 meters above sea level.

The Bluethroat population in Øvre Heimdalen has been thoroughly studied since 1991. It is a common migrant breeding in the study area, with an estimated breeding density of 38 pairs per km² (Anthonisen *et al.* 1997). The Willow Warbler is a very common migrant that breeds quite densely in the study area, approximately 140 pairs per km² (Bjørnstad & Lifjeld 1996).

2.2. Field procedures

Adult males of both species were caught in their territories using mist nets and playback, and transported into a lab building. The Bluethroat males were caught during the breeding season of 2004 (between 1 and 21 June), and the Willow Warblers during the breeding season of 2002 (between 12 and 29 May). In the lab, the birds were measured, blood sampled and sacrificed by cervical dislocation. Blood was sampled by brachial venipuncture for inclusion in the tissue collection at the Natural History Museum, University of Oslo. After dissection, the seminal glomera from both species were stored in 3% glutaraldehyde and squeezed in the solution so that sperm could rapidly be diluted and fixed in the medium for later morphometric analyses.

2.3. Sperm morphometry measurements

A droplet (approximately 3 µl) of fixed sperm was applied on a microscope slide. We used a Leica DC500 camera mounted on a Leica DM6000 B light microscope to take digitalized photographs of spermatozoa at a magnification of 320 ×. Abnormal spermatozoa (broken tail, damaged or missing acrosome) were not used. Using a line-chain tool in the Leica IM1000 software, we measured the total sperm length from the anterior tip of the acrosome to the end of the flagellum on the digital images. We measured 30 spermatozoa per individual in the Bluethroat and 20 spermatozoa per individual in the Willow Warbler. For a single Willow Warbler male, the same 20 spermatozoa were measured blindly twice, and the measurements were highly repeatable ($r = 0.99$, $F_{19,20} = 152.72$, $P < 0.001$; Lessells & Boag 1987). All sperm measurements were conducted by T.L.

2.4. Statistical methods

We used a resampling procedure to illustrate how the accuracy of mean sperm length is influenced by the number of males measured. From our samples of 46 males in each species we randomly selected a given number of males, calculated their mean sperm length and repeated the procedure 1,000 times. This was done for all sample sizes between 1 and 30 males. Further, we applied similar resampling procedures, on the between-male level, to illustrate how the spread in estimated CV is influenced by the number of males sampled, and on the within-male level, to illustrate how the spread in CV is influenced by the number of spermatozoa measured. This was done for sample sizes between 2 and 30 males and 2 and 30 spermatozoa respectively. All resampling was done with replacement and were conducted using Resampling Stats for Excel 3.2 (Resampling Stats, Inc.) and Microsoft® Office Excel 2003 (Microsoft® Corporation). All other statistical analyses were performed using STATISTICA version 7.1 (StatSoft, Inc). Graphs were constructed using Origin® v7.0300 (OriginLab Corporation).

Table 2. Descriptive statistics for sperm length (μm) in Bluethroats and Willow Warblers. Data derived from 1380 spermatozoa from 46 Bluethroat males and 920 spermatozoa from 46 Willow Warbler males (30 and 20 spermatozoa per individual, Bluethroat and Willow Warbler respectively).

| Species | Mean sperm length \pm SD (CV ¹) | Range (min–max) | Mean intra-male CV |
|----------------|---|-----------------|--------------------|
| Bluethroat | 216.43 \pm 6.09 (2.81) | 197.40–232.83 | 1.43 |
| Willow Warbler | 94.48 \pm 2.35 (2.49) | 89.08–99.60 | 1.46 |

1. CV = coefficient of variation calculated as SD/mean*100

3. Results

The Bluethroat sperm were more than twice as long as the Willow Warbler sperm (Table 2). The mean CV of within-male sperm length was considerably lower than between-male CV of sperm length in both species (Table 2). Hence, there was a significant variation in mean sperm length among males, and a high repeatability of sperm length for individual males (Bluethroat: $r = 0.79$, $F_{45,1334} = 112.49$, $P < 0.001$; Willow Warbler: $r = 0.73$, $F_{45,874} = 54.84$, $P < 0.001$). This is also apparent when plotting mean sperm length \pm SD for each of the males (Fig. 1). Individual male mean sperm lengths were normally distributed in both species (Shapiro-Wilks W tests: Bluethroat: $W = 0.98$, $P = 0.43$, $n = 46$ males; Willow Warbler: $W = 0.99$, $P =$

0.98, $n = 46$ males). A resampling procedure illustrates how the spread in estimates of mean sperm length is reduced with the number of males measured (Fig. 2).

We ran a similar resampling procedure on the spread in between-male CV, illustrating that CV can well be under- or overestimated when few males are sampled (Fig. 3). Further, we obtained resampling estimates of within-male CV for two individuals of each species, the one with the lowest and the one with the highest CV, respectively (Fig. 4).

The simulations reveal a general pattern of rapid decline in the spread of estimates for small sample sizes. Beyond a sample size of 10 spermatozoa, there is only a marginal decrease in the spread of estimated CV values. The simulations

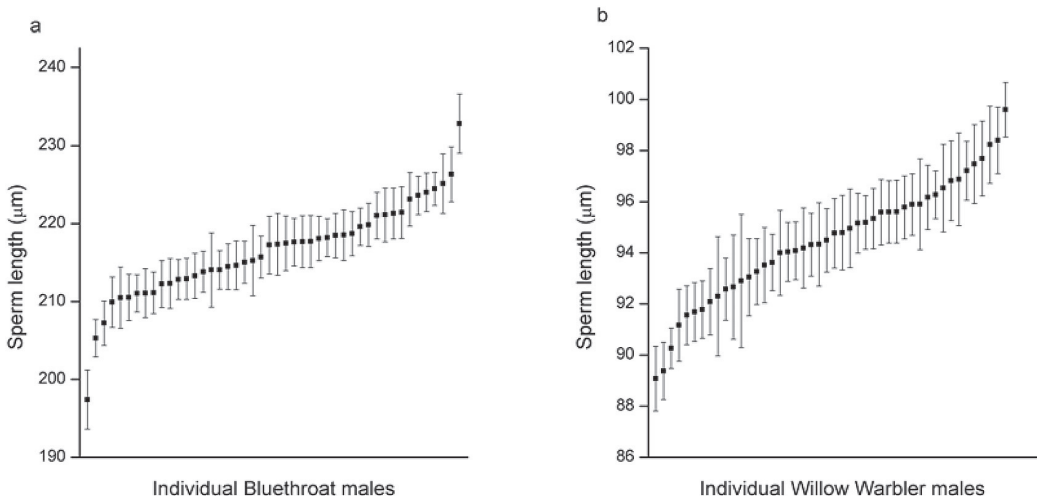


Fig. 1. Estimates of mean sperm length \pm SD (μm) of individual Bluethroat (a) and Willow Warbler (b) males, sorted by mean length. Calculations were based on 30 spermatozoa per individual in the Bluethroat and 20 spermatozoa per individual in the Willow Warbler.

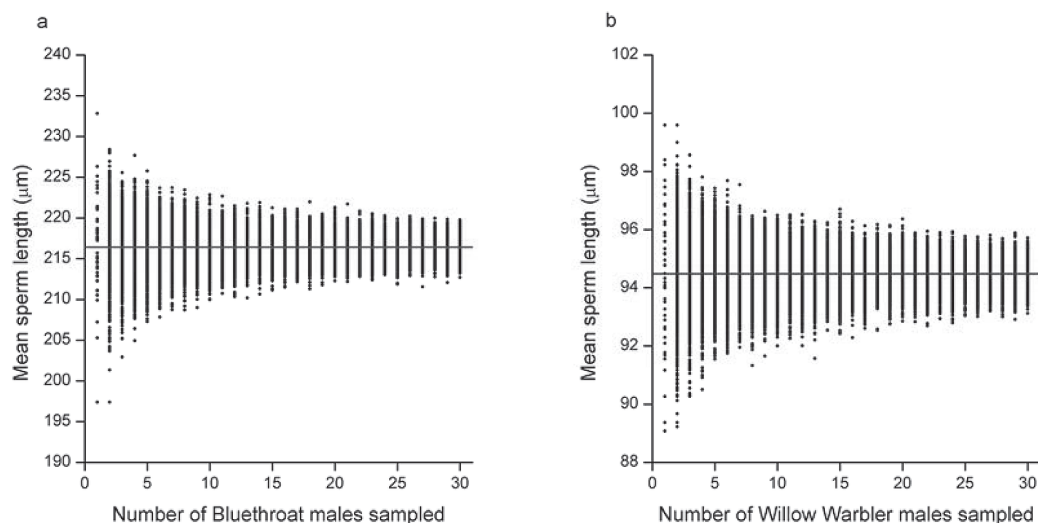


Fig. 2. Estimates of mean sperm length of Bluethroats (a) and Willow Warblers (b) as a function of the number of males sampled. Values are calculated from resampling (1,000 iterations per sample size) of the mean sperm length of 46 males in each species. The straight line indicates the overall mean sperm length (mean of means).

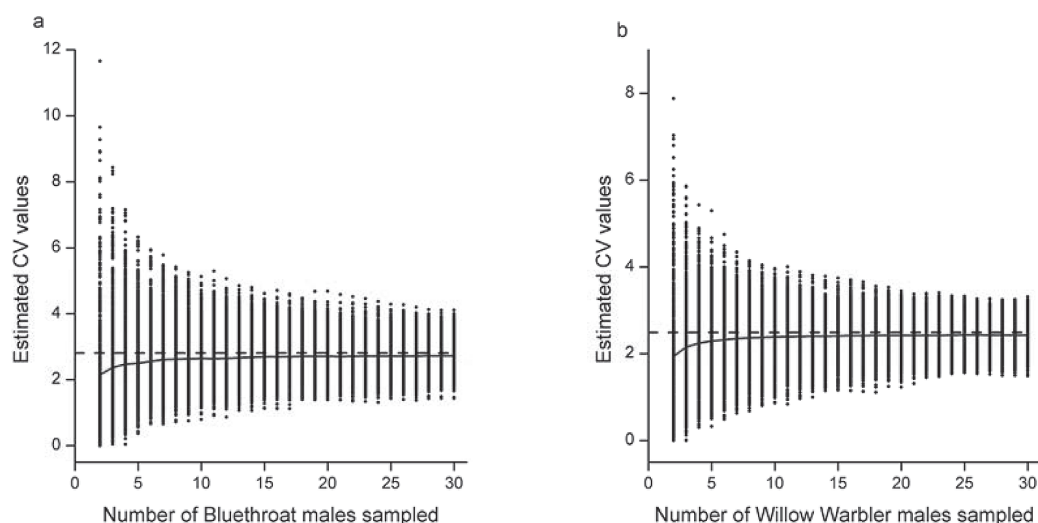


Fig. 3. Estimates of inter-male coefficient of variation (CV) of sperm length in Bluethroats (a) and Willow Warblers (b) as a function of the number of males sampled (2–30 males). Values calculated from resampling (1,000 iterations per sample size). The dashed line indicates the mean inter-male CV, the continuous line indicates the mean CV calculated from the simulations.

also revealed a general underestimation of CVs at small samples sizes, as illustrated by the lines for average CV (Fig. 3 and 4). Hence, CV values should be adjusted for sample size, especially if the sample size is low, as suggested by Sokal and Rohlf (1995).

4. Discussion

In the present study we have demonstrated a significant variation in sperm length at the intraspecific level in the Bluethroat and the Willow Warbler, and that sperm are more variable between than

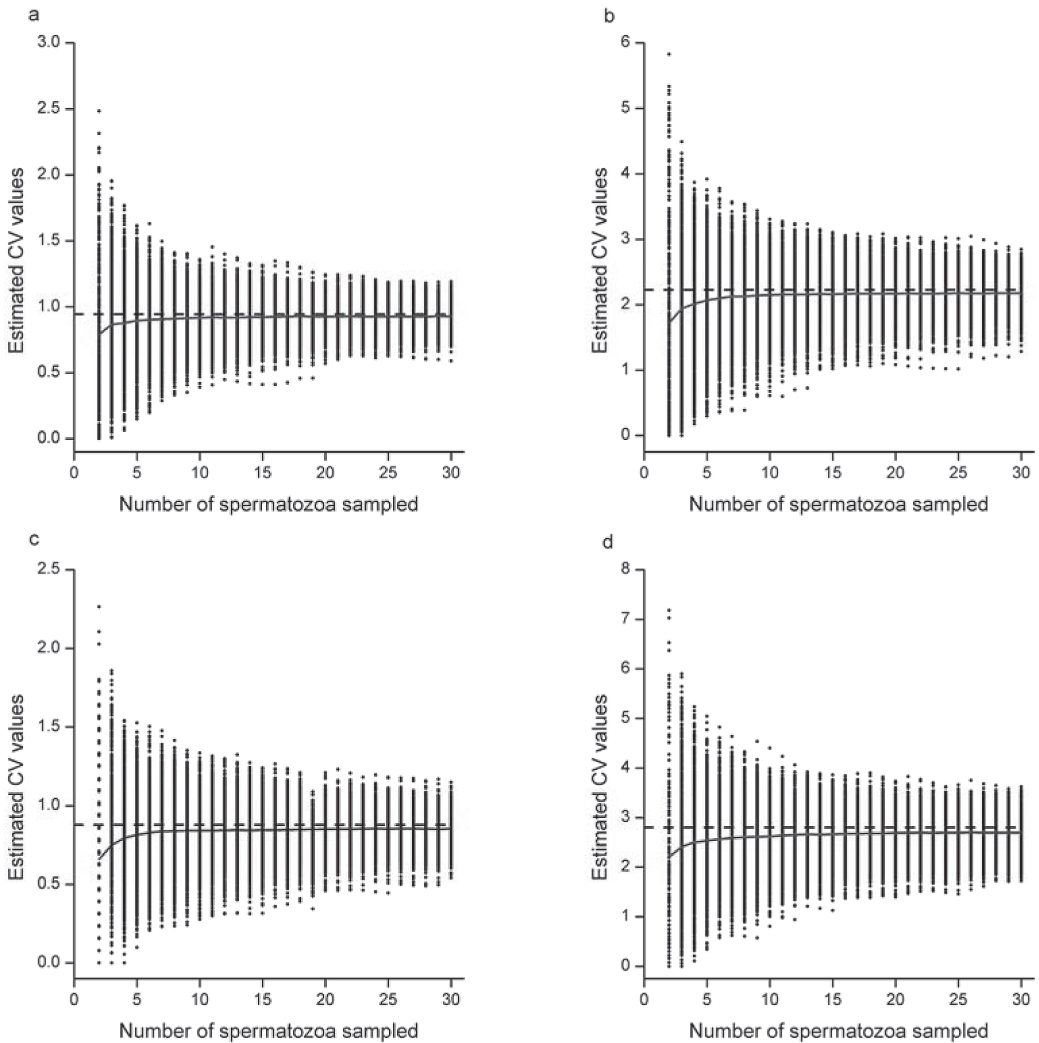


Fig. 4. Estimates of intra-male coefficient of variation (CV) of sperm length in the Bluethroat showing lowest CV (a), the Bluethroat showing highest CV (b), the Willow Warbler showing lowest CV and the Willow Warbler showing highest CV (d), as a function of the number of spermatozoa sampled (2–30). Values calculated from resampling (1,000 iterations per sample size). The dashed line indicates the intra-male CV, the continuous line indicates the mean CV calculated from the simulations.

within males in both species. Consequently, for adequate estimates of mean sperm length, it seems more important to measure several males per species than several spermatozoa per male. In addition, we illustrate how the spread in estimates of mean sperm length and CV values is reduced when sample size increases. Interestingly, both species show more or less the same pattern with just a mar-

ginal improvement of estimates beyond ten males (Fig. 2 and 3). It is important to note that these simulations are based on the observed sperm lengths in our two study species, and generality can therefore not be claimed.

There are some reports on between-male variation in sperm length in birds (e.g. Allen *et al.* 1968, Birkhead & Fletcher 1995, Tuttle *et al.* 1996, Birk-

head *et al.* 1997, Birkhead *et al.* 2005, Birkhead *et al.* 2006), and published information on sperm length is available for about 120 bird species (Briske & Montgomerie 2007). However, as far as we know, there is only one previous study that has reported both between-male and within-male variation in sperm lengths in birds. Dixon & Birkhead (1997) measured the length of 30 spermatozoa in each of four Reed Buntings and reported average sperm length \pm SD for each male, hence CV could easily be calculated. The mean within-male CV in sperm length amounted to 1.89, whereas the between-male CV was only 0.51. Hence, in that species there was a considerably higher within-male variance than between-male variance in sperm length, that is, a pattern opposite to what we found in Bluethroats and Willow Warblers. However, it is unclear whether the result in Dixon & Birkhead (1997) reflects the actual intraspecific variation, or is an artifact of the low sample size. In fact, our resampling procedures illustrate how CV is likely to be underestimated when the sample size is low (Fig. 3 and 4).

From these examples, it is obvious that both levels of intraspecific variation in sperm length, i.e. between-male and within-male, are relevant and important for characterizing intraspecific variation in sperm length and for sample size assessments (number of males and spermatozoa per male to be measured). We would therefore recommend future studies to report both the within-male and the between-male variance in sperm length, as well as the number of sperm measured per male, the number of males examined and the overall mean sperm length (mean of means). We found high repeatabilities for sperm length within ejaculates, but our data did not allow us to investigate repeatability between ejaculates. However, high between-ejaculate repeatability has been demonstrated in the Zebra Finch (*Taeniopygia guttata*) (Birkhead & Fletcher 1995), hence obtaining one ejaculate should give an adequate estimate of individual sperm length.

Notably, sperm samples can easily be obtained from wild birds either through gently massaging the cloacal protuberance of males in breeding condition (Wolfson 1952) or through fecal sampling (Immler & Birkhead 2005). Indeed, Briskie & Montgomerie (2007) encourages field ornithologists to consider including sperm sampling as a

routine procedure when handling male birds during the breeding season.

Birkhead *et al.* (2005) hypothesized that sperm competition may select for lower variation in sperm traits. Indeed, a negative relationship between indices of sperm competition risk and variation in sperm length and other sperm traits has recently been documented in passerines (Calhim *et al.* 2007, own unpublished data). Calhim *et al.* (2007) suggests that sperm competition may enforce stabilizing selection on sperm size variation through selection against the extreme sperm sizes.

In the present study, we have shown that variation in sperm length is considerably lower within males as compared to between males in Bluethroats and Willow Warblers. Furthermore, our resampling simulations suggest that sampling a minimum of 10 males and measuring a minimum of 10 spermatozoa per male will give adequate estimates of both within- and between-male sperm length in these species. Future studies on more species will establish whether our findings in the present study reflect general patterns of intraspecific variation in sperm length in birds.

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Lajinsisäistä vaihtelua pajulinnun ja sinirinnan siittiöiden pituudessa

Siittiöiden morfologiaa linnuilla on tutkittu paljon ja niiden muoto vaihtelee huomattavasti eri lajien välillä. Aiemmistä tutkimuksista huolimatta ei lajinsisäisestä siittiöiden koon vaihtelusta tiedetä paljoakaan. Analysoimme koiraiden välisen ja yksilöllisen vaihtelun siittiöiden kokonaispituudessa sinirinnoilla ja pajulinnulla. Molemmilla lajeilla koiraiden välinen vaihtelu siittiöiden pituudessa oli lähes kaksi kertaa niin suurta kuin yksilöllinen vaihtelu. Yksittäisten lintujen siittiöiden pituuksien toistettavuus mittausten välillä oli erittäin suur-

ta (sinirinta: $r = 0.73$, pajulintu: $r = 0.79$). Näytämme tutkimuksessamme, kuinka mitattujen koiraiden määrä vaikuttaa sukusolujen pituuden arviointuun vaihteluväliin ja muuntelukertoimeen (CV). Lisäksi näytämme kuinka yksilöllinen siittiöiden pituuden CV muuttuu lisääntyvien mittausten mukana. 10 koiraan ja 10 sukusolun mittaaminen koirasta kohden antaa riittävän yksilöllisen ja koiraiden välisen siittiöiden pituuden ja muuntelukertoimen molemmille lajeille.

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